Knockdown of Beclin 1 inhibits vitamin K3-induced autophagy, but promotes apoptosis of human hepatoma SMMC-7721 cells

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Abstract. The aim of the present study was to investigate the effects of Beclin 1 knockdown on spontaneous and vitamin K_3 (VK_3)-regulated autophagy, survival and apoptosis in human hepatocarcinoma SMMC-7721 cells, and to explore the potential mechanisms underlying the action of Beclin 1 knockdown in the processes of autophagy and apoptosis. A recombinant plasmid-expressing small interfering RNA (siRNA) targeting Beclin 1 mRNA was constructed and introduced into SMMC-7721 cells. The expression of Beclin 1 was determined by reverse transcription-polymerase chain reaction and Western blotting. Subsequently, the impact of Beclin 1 knockdown on spontaneous and VK_3-induced autophagy, survival and apoptosis was determined. The expression of cyclin D1, cyclin-dependent kinase 4 (CDK4), Bcl-2, Bcl-xL and the activation of caspase-3 were examined by Western blotting. Transfection with the plasmid for Beclin 1 siRNA expression dramatically down-regulated Beclin 1 expression in SMMC-7721 cells. The knockdown of Beclin 1 expression significantly inhibited spontaneous and VK_3-induced autophagy, but did not affect spontaneous proliferation and apoptosis in SMMC-7721 cells *in vitro*. By contrast, the silencing of Beclin 1 expression significantly enhanced the inhibition of survival and proliferation by VK_3, and promoted VK_3-induced apoptosis by significantly down-regulating cyclin D1, CDK4, Bcl-2 and Bcl-xL expression and enhancing caspase-3 activation in SMMC-7721 cells *in vitro*. Our data indicate that Beclin 1 is a positive regulator of autophagy, but a negative regulator of VK_3-induced apoptosis in human hepatoma cells.

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Introduction

Vitamin K_3 (2-methyl-1,4-naphthoquinone; VK_3), a synthetic version of vitamin K, is a potent inducer of oxidative stress. Numerous studies have demonstrated that VK_3 inhibits the growth of multiple types of tumor cells both *in vitro* and *in vivo* (1-3). VK_3 induces G1 cell cycle arrest by down-regulating the expression of cyclin D1 and cyclin-dependent kinase 4 (CDK4) in hepatocellular carcinoma cells (1,4). Other studies have indicated that VK_3 induces the caspase-dependent apoptosis of tumor cells (5-7). Recently, vitamin K2-induced cell growth inhibition was attributed to the induction of autophagy formation in cholangiocellular carcinoma cells (8). However, the mechanisms underlying VK_3-mediated tumor cell growth inhibition are poorly understood. Whether VK_3 induces autophagy, and how the regulators of autophagy affect cell survival, proliferation and apoptosis in hepatocarcinoma cells have never been explored.

Apoptotic and autophagic cell death are two distinct forms of programmed cell death, which is closely associated with the development and progression of many diseases, such as neurodegenerative and autoimmune diseases, malignant tumors and pathogenic microorganism infections (9,10). Cells undergoing apoptosis exhibit cell shrinkage, membrane blebbing, nuclear fragmentation, formation of apoptotic bodies, mitochondrial release of cytochrome c and massive caspase activation (11). By contrast, autophagic cell death is characterized by massive vacuolization in the cytoplasm with accumulation of autophagic vacuoles (AVs), but without chromatin condensation (12). During the autophagic process, some cytoplasmic components and organelles are sequestered within double-membrane vesicles, termed autophagosomes, and delivered to lysosomes for degradation. The degradation products are reused in cells, and these processes regulate the cell growth, embryonic development and pathogenesis of many diseases (13). Apparently, certain stimuli may activate the signaling pathways involved in both autophagy and apoptosis (14). Therefore, the discovery of molecules that regulate both autophagy and apoptosis may be of great significance in the development of drugs for the treatment of cancer.

Beclin 1, the mammalian homologue of yeast Atg6/Vps30, is a haploinsufficient tumor suppressor (15). Mice carrying heterozygous disruption of the Beclin 1 gene suffer from a high incidence of spontaneous tumors, including hepatocellular carcinoma (16). Allelic loss of the Beclin 1 gene is commonly found in many of human malignant tumors, such as breast, ovarian and prostate cancer (17). Beclin 1 is an upstream critical regulator of various autophagy signaling pathways, and is responsible for mediating the formation of AVs (18). Moreover, the knockdown of Beclin 1 expression enhances Fas-induced apoptosis and doxorubicin-mediated aggravating mitochondrial permeabilization in HepG2 cells *in vitro* (19), while the overexpression of Beclin 1 augments the cis-diamminedichloroplatinum-induced apoptosis of gastric cancer MKN28 cells by enhancing caspase-9 activity (20). Therefore, Beclin 1 is a dual functional regulator of autophagy and apoptosis. However, whether Beclin 1 regulates VK_3-mediated tumor cell growth inhibition has not been investigated.

This study aimed to examine the effects of Beclin 1 silencing on VK_3-induced autophagy and apoptosis in human hepatoma SMMC-7721 cells, and to determine the mechanisms underlying the action of Beclin 1 in VK_3-induced autophagy and apoptosis.

Materials and methods

Cell culture and transfection. The human hepatoma cell line SMMC-7721 was maintained in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) at 37˚C in a 5% CO_2 incubator.

The specific small interfering RNA sequences were designed for targeting the human Beclin 1 gene (at position 515-533; Genbank accession no. NM_003766) according to the following DNA sequences: forward, 5'-GATCCGTGA ATGAGGATGACAGTGATTCAAGAGATCACTGTCATC CTCATTCATTTTTTGGAAA-3' and reverse, 5'-AGCTTT TCCAAAAAATGAATGAGGATGACAGTGATCTCTTGA ATCACTGTCATCCTCATTCACG-3', The sequences were then cloned into the plasmid of pSilencer 3.1 H1-neo (Austin, USA) to generate the plasmid of pSilence-Beclin 1. Upon reaching 80% confluence in 6-well plates, the SMMC-7721 cells were transfected with pSilence-Beclin 1 or vehicle control, respectively, using Lipofectamine 2000 Plus reagent (Invitrogen) according to the manufacturer's instructions, and cultured for 48 h. The cells were then trypsinized and harvested. The expression of Beclin 1 in the transfected and untransfected control cells was determined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting.

Methyl thiazolyl tetrazolium (MTT) assay. SMMC-7721 cells that had been transfected with pSilence-Beclin 1 or vehicle at $1x10^4$ cells/well were cultured in quintuplicate in 10% FBS IMDM in 96-well plates in the presence or absence of 40 μM VK_3 (Sigma, USA) at 37˚C for 18 h. During the last 4 h of culture, the cells were exposed to 1 mg/ml MTT and the generated formazan was dissolved in 150 μl DMSO. The absorbance was then measured at 570 nm. Cell viability was expressed as a percentage, and the viability of the cells transfected with vehicle and cultured in the absence of VK_3 was designated as 100%.

Reverse transcription-polymerase chain reaction. Total RNA was isolated from unmanipulated control SMMC-7721 cells and from cells transfected with pSilence-Beclin 1 or empty vehicle using TRIzol Reagent, according to the manufacturer's instructions (Invitrogen). Total RNA was reverse transcribed into cDNA using the Superscript® VILO™ cDNA Synthesis kit (Invitrogen). The levels of Beclin 1 mRNA transcripts in these groups of cells were determined by PCR, and the resulting cDNA was used as the template and specific primers. The sequences of primers were forward, 5'-ACCGCAAGATAGTGGCAGAA-3' and reverse, 5'-GCGACCCAGCCTGAAGTTAT-3' for Beclin 1; while forward, 5'-GGGTGATGCTGGTGCTGAGTATGT-3' and reverse, 5'-AAGAATGGGTGTTGCTGTTGAAGTC-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After 30 cycles of amplification, the PCR products were resolved by agarose gel electrophoresis.

Monodansylcadaverine (MDC) staining. To examine the impact of Beclin 1 knockdown on the autophagic process, the contents of AVs were determined by MDC staining and FACS analysis (21). Briefly, the pSilence-Beclin 1-transfected and vehicle-transfected SMMC-7721 cells at $2x10^5$ cells/well were cultured in triplicate in 6-well plates in the presence or absence of VK_3 (40 μM) for 12 h. The cells were then stained with 10 μM MDC (Sigma) at 37˚C for 40 min and fixed with 4% paraformaldehyde for 15 min. After washing twice with PBS, the intensity of MDC staining in the cells was characterized by FACS analysis on a flow cytometer (Becton Dikinson) at an excitation wavelength of 488 nm. A total of 10,000 cells was measured in each group.

Hoechst 33342 staining. To examine the impact of Beclin 1 knockdown on VK_3-induced apoptosis, the pSilence-Beclin 1-transfected and vehicle-transfected SMMC-7721 cells at $2x10^5$ cells/well were cultured on coverslips in 6-well plates. Upon reaching 80% confluence, the cells were treated in triplicate with or without 40 μM VK_3 for 12 h and fixed with 4% paraformaldehyde. After washing with PBS, the cells were stained with Hoechst 33342 (10 μg/ml) (Sigma) for 15 min, and the coverslips were mounted onto glass slides. Chromatin condensation in different groups of cells was observed under a confocal laser microscope (Olympus FV1000). The number of apoptotic cells was counted in a blinded fashion.

Apoptosis assay. The impact of Beclin 1 knockdown on VK_3-induced cell apoptosis was determined by FACS analysis. The pSilence-Beclin 1-transfected and vehicle-transfected SMMC-7721 cells at $2x10^5$ cells/well were cultured in triplicate in 6-well plates in the presence or absence of 40 μM VK_3 for 12 h. Subsequently, the cells were harvested and fixed with cold ethanol (70% w/v) for 24 h. After washing with PBS, the cells were digested with 1% RNase A at 37˚C for 30 min and stained with 50 μg/ml propidium iodide (PI; Invitrogen) at 4˚C for 30 min. The cellular DNA contents were determined by FACS analysis using CellQuest software. Sub-diploid cells were considered apoptotic.

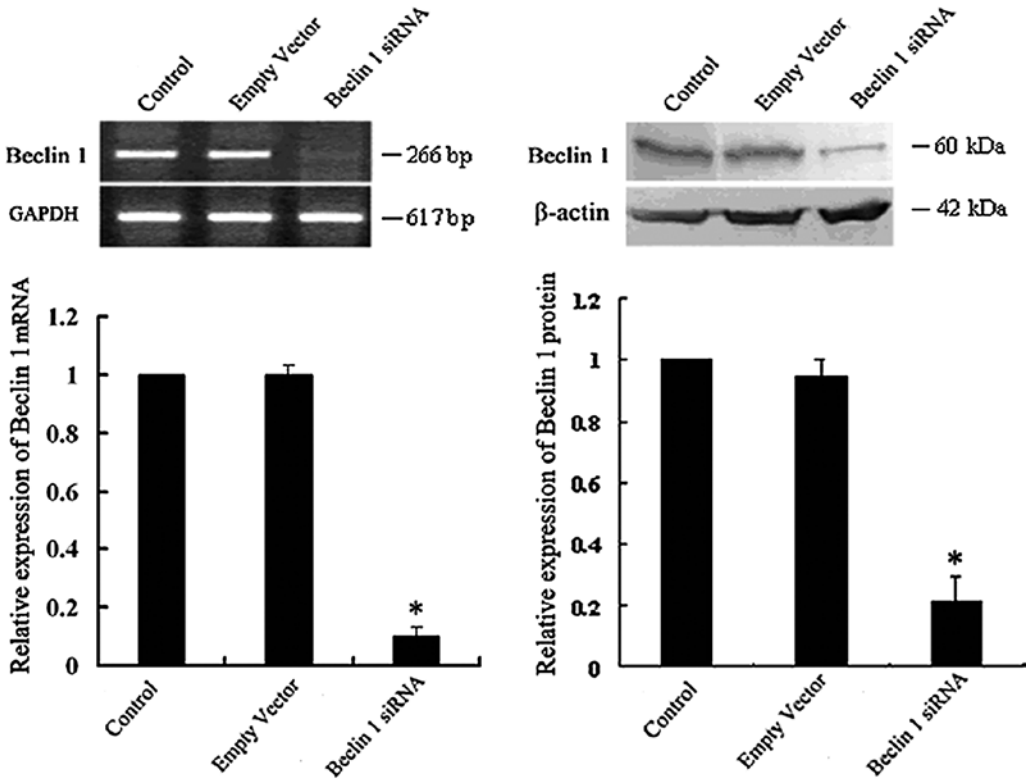

Figure 1. Knockdown of Beclin 1 expression in SMMC-7721 cells. SMMC-7721 cells were transfected with or without vehicle plasmid pSilencer 3.1 or pSilence-Beclin 1 for 48 h. The levels of Beclin 1 mRNA transcripts and protein expression were determined by RT-PCT and Western blotting, followed by semi-quantitative analysis using densitometric scanning (with Beclin 1 expression in untransfected cells designated as 1). Images are representative, and data are expressed as the mean ± SD of Beclin 1 expression relative to control GAPDH or β-actin from three independent experiments. *P<0.01 vs. the control group, determined by the Student's t-test.

Western blotting. After treatment with or without VK_3 (40 μM) for 12 h, the cells were harvested and the cell lysates were prepared, followed by the determination of protein concentrations using the Bradford assay. The relative levels of proteins in these groups of cells were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Individual cell lysates (30 μg/lane) were separated by 12% SDS-gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk for 1 h and washed three times with PBS Tween-20 (PBST). The target proteins were probed with individual antibodies against Beclin 1, cyclin D1, CDK4, Bcl-2, Bcl-xL, cleaved caspase-3 and internal control β-actin (Santa Cruz Biotechnology, USA) overnight at 4˚C, respectively. The bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2,000) (Pierce, USA), with gentle agitation at room temperature for 1 h, and visualized using diaminobenzidine and the Tanon gel imaging system. The levels of each target protein relative to control β-actin were determined by densitometric scanning.

Statistical analysis. Data shown are expressed as the mean ± SD. Differences between groups of cells were determined by one-way analysis of variance, followed by the Tukey *post-hoc* test. Differences between two groups were analyzed by the Student's t-test. A P-value of <0.05 was considered statistically significant.

Results

Knockdown of Beclin 1 expression in SMMC-7721 cells by Beclin 1 siRNA. Beclin 1 is a critical regulator of the survival, autophagy and apoptosis of many types of cells. To determine the impact of Beclin 1 knockdown on the proliferation and apoptosis of human hepatocarcinoma cells, the siRNA sequences specifically targeting the Beclin 1 gene were designed and cloned into pSilencer to generate the pSilence-Beclin 1 plasmid. Following transfection with pSilence-Beclin 1 or empty vehicle, the levels of Beclin 1 mRNA transcripts and protein expression were determined by RT-PCR and Western blotting. As shown in Fig. 1, the transfection of SMMC-7721 cells with vehicle did not affect the expression of Beclin 1, evidenced by similar levels of Beclin 1 expression in unmanipulated and vehicle-transfected SMMC-7721 cells. By contrast, transfection with pSilence-Beclin 1 dramatically reduced the levels of Beclin 1 mRNA transcripts and protein expression in SMMC-7721 cells. These data demonstrate that the expressed siRNA effectively silenced Beclin 1 expression in SMMC-7721 cells.

Knockdown of Beclin 1 expression inhibited spontaneous and VK_3-induced autophagy in SMMC-7721 cells. Spontaneous autophagy is a catabolic process in the cell survival and growth. During the process of autophagy, intracellular organelles and long-lived proteins form the double-membrane

Table I. Intensity of MDC staining in SMMC-7721 cells.

Group	Fluorescence intensity of MDC staining
Control	103.2±8.6
Beclin 1 siRNA	33.2±8.6[a]
VK3	691.5±19.5[a]
VK3 + Beclin 1 siRNA	138.3±9.8[b]

Data are expressed as the mean ± SD of each group of cells from three independent experiments. [a]P<0.01 vs. the control group (vehicle-transfected cells without VK_3 treatment); [b]P<0.01 vs. the VK3 group.

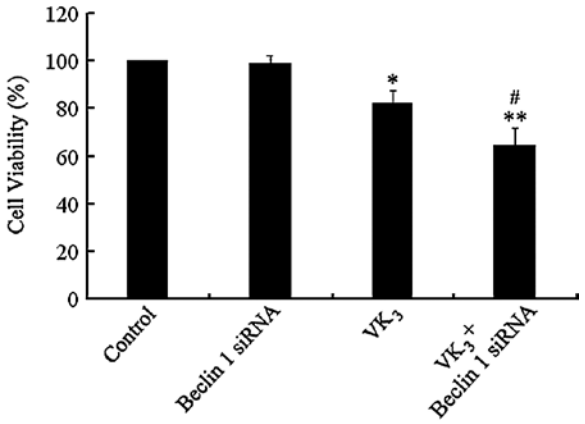

Figure 2. Knockdown of Beclin 1 expression enhances the inhibition of the proliferation of SMMC-7721 cells by VK_3. SMMC-7721 cells were transfected with or without the vehicle plasmid or pSilence-Beclin 1, and the effects of VK_3 on the proliferation of these cells were determined by the MTT assay. Data are expressed as the mean ± SD of each group of cells from five independent experiments, and the proliferation of vehicle-transfected cells without VK_3 treatment (control) was designated as 100%. The proliferation of control cells was similar to that of unmanipulated SMMC-7721 cells (data not shown). *P<0.05 vs. the control group; **P<0.01 vs. the control group; #P<0.01 vs. the VK3 group.

vesicles of AVs, which can be selectively stained by MDC. To determine the impact of Beclin 1 knockdown on the spontaneous and VK_3-induced autophagic process, pSilence-Beclin 1-transfected and vehicle-transfected control cells were treated with or without VK_3 for 12 h, and the accumulation of AVs in SMMC-7721 cells was determined by MDC staining. As shown in Table I, the intensity of MDC staining in the Beclin 1-silenced cells was significantly lower than that in the vehicle-transfected controls (P<0.01). Following treatment with VK_3, there was an approximately 6-fold increase in the number of control cells undergoing the autophagic process, and significantly increased levels of MDC staining were detected in VK_3-treated control cells, indicating that VK_3 promoted the autophagic process in SMC-7721 cells *in vitro*. By contrast, the average intensity of MDC staining in VK_3-treated Beclin 1-silenced cells was dramatically reduced, as compared to that in VK_3-treated control cells. Collectively, the data indicated that the knockdown of Beclin 1 expression inhibited the spontaneous and VK_3-promoted autophagic process in SMMC-7721 cells *in vitro*.

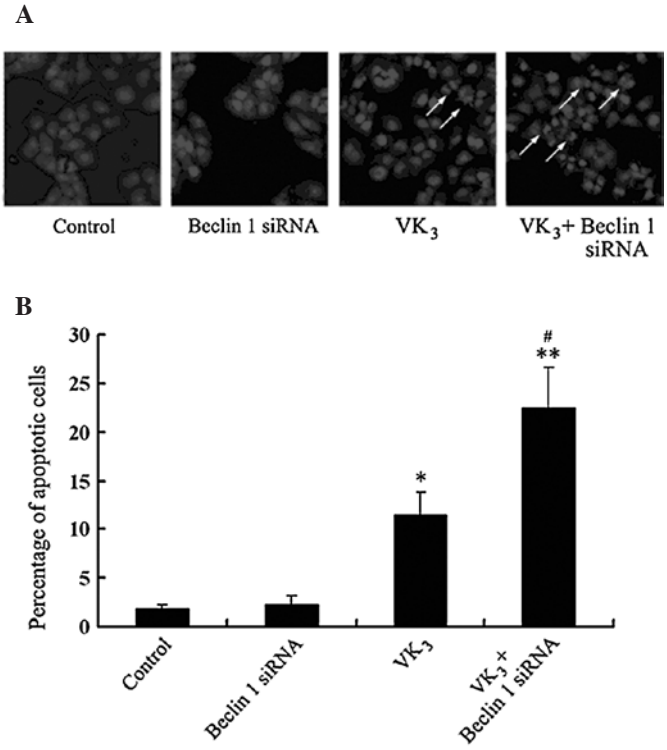

Figure 3. Knockdown of Beclin 1 expression enhances VK_3-induced apoptosis in SMMC-7721 cells. The vehicle-transfected control and Beclin 1-silenced SMMC-7721 cells were treated with or without VK_3, and the apoptotic cells were characterized by Hoechst 33342 staining. Images are representative, and data are expressed as the mean ± SD of each group of cells from three independent experiments. *P<0.05 vs. the control group; **P<0.01 vs. the control group; #P<0.01 vs. the VK_3 group.

Knockdown of Beclin 1 expression enhanced VK_3-induced inhibition of SMMC-7721 cell proliferation. The MTT assay was used to examine whether the knockdown of Beclin 1 affects the inhibition of the survival and proliferation of SMMC-7721 cells by VK_3 (Fig. 2). It was observed that the percentage survival of Beclin 1-silenced cells was similar to that of vehicle-transfected controls cells, suggesting that the knockdown of Beclin 1 expression did not significantly affect the survival and proliferation of SMMC-7721 cells *in vitro*. Notably, treatment with VK_3 reduced the overall survival rates of vehicle-transfected cells by ~20%. The same treatment decreased the survival rates of Beclin 1-silenced cells to ~60%, which was significantly lower than in the VK_3-untreated control cells (P<0.01). These data indicate that the knockdown of Beclin 1 expression enhanced the inhibitory effect of VK_3 on the survival and proliferation of SMMC-7721 cells *in vitro*.

Knockdown of Beclin 1 expression promotes VK_3-induced apoptosis of SMMC-7721 cells. To determine potential mechanisms of Beclin 1, Hoechst 33342 staining was used to examine whether the knockdown of Beclin 1 expression regulates the spontaneous and VK_3-induced apoptosis of SMMC-7721 cells (Fig. 3). Very few cells were observed to undergo spontaneous apoptosis, as evidenced by the chromatin-condensed nuclei, regardless of whether the cells had been transfected with the vehicle or pSilence-Beclin 1. This indicated that the knockdown of Beclin 1 expression did not promote the spontaneous apoptosis of SMMC-7721 cells under the experi-

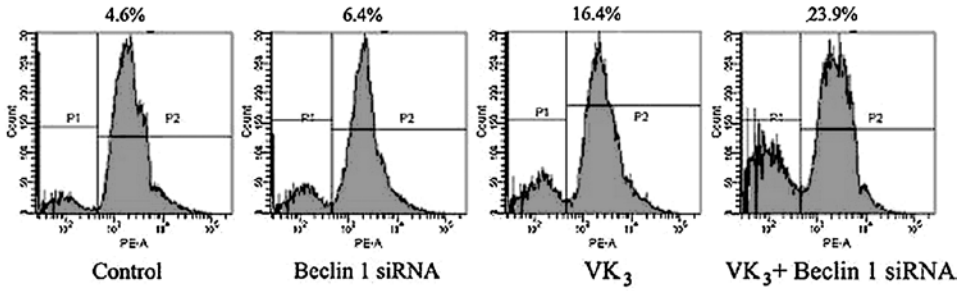

Figure 4. Flow cytometric analysis of spontaneous and VK_3-induced apoptosis in SMMC-7721 cells. The vehicle-transfected control and Beclin 1-silenced SMMC-7721 cells were treated with or without VK_3 and stained with PI. Apoptotic cells were characterized by flow cytometric analysis. Data are representative of each group of cells, and the P1 phase represents apoptotic cells.

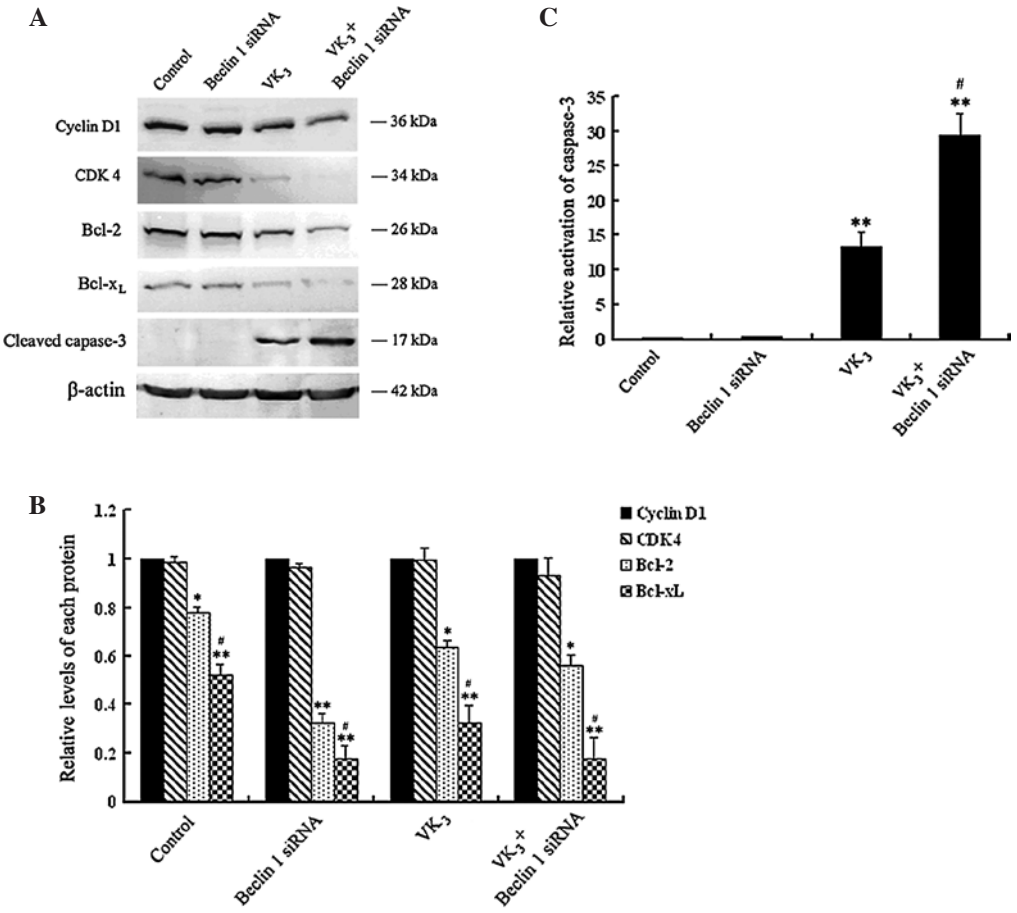

Figure 5. Knockdown of Beclin 1 expression modulates the VK_3-induced down-regulation of cyclin D1, CDK4, Bcl-2 and Bcl-xL expression and active caspase-3 in SMMC-7721 cells. The vehicle-transfected control and Beclin 1-silenced SMMC-7721 cells were treated with or without VK_3, and the expression of cyclin D1, CDK4, Bcl-2, Bcl-xL and active caspase-3 was determined by Western blotting using specific antibodies. The relative levels of each protein to control β-actin in individual groups of cells were determined by densitometric scanning. (A) Representative images. (B) Semi-quantitative analysis of protein expression. The levels of each protein in control cells were designated as 1. (C) Semi-quantitative analysis of active caspase-3. The levels of active caspase-3 in control cells were designated as 100%. Data are expressed as the mean ± SD of the levels of each protein relative to control β-actin. $^{*}P<0.05$ vs. the control group; $^{**}P<0.01$ vs. the control group; $^{\#}P<0.01$ vs. the VK_3 group.

mental conditions. Quantitative analysis of apoptotic cells revealed that the frequency of apoptotic cells was significantly higher in VK_3-treated control cells than in untreated controls ($P<0.05$). Furthermore, the frequency of apoptotic VK_3-treated Beclin 1-silenced SMMC-7721 cells was significantly elevated, by 200%, as compared to VK_3-treated vehicle-transfected control cells (Fig. 3B). Similar results were observed using PI staining and flow cytometry analysis (Fig. 4). While there was no significant difference in the number of apoptotic cells between vehicle-transfected and Beclin 1-silenced SMMC-7721 cells (4.6±0.7 vs. 6.4±0.9%, $P>0.05$), treatment with VK_3 significantly induced the apoptosis of both vehicle-transfected and Beclin 1-silenced SMMC-7721 cells. Notably, the number of apoptotic VK_3-treated Beclin 1-silenced SMMC-7721 cells was significantly higher than that of apoptotic VK_3-treated vehicle-treated controls (23.9±5.4 vs. 16.4±2.7%, $P<0.01$).

Apparently, the knockdown of Beclin 1 expression enhanced the VK_3-induced apoptosis of SMMC-7721 cells *in vitro*.

Knockdown of Beclin 1 expression regulates VK_3-modulated expression of cyclin D1, CDK 4, Bcl-2 and Bcl-xL proteins and caspase-3 activation in SMMC-7721 cells. To further determine the molecular mechanisms by which Beclin 1 silencing regulated VK_3-modulated cell survival and apoptosis, cyclin D1, CDK4, Bcl-2 and Bcl-xL expression as well as caspase-3 activation were determined by Western blotting. First, the knockdown of Beclin 1 expression appeared to affect neither the expression of cyclin D1, CDK4, Bcl-2 or Bcl-xL, nor to induce caspase-3 activation, since the relative levels of cyclin D1, CDK4, Bcl-2 and Bcl-xL in Beclin 1-silenced cells were indistinguishable from those in vehicle-transfected cells, and since there were no significantly elevated levels of caspase-3 activation in either of the groups of cells (Fig. 5). Furthermore, treatment with VK_3 significantly inhibited the expression of cyclin D1, CDK4, Bcl-2 and Bcl-xL, accompanied by dramatically elevated levels of caspase-3 activation in vehicle-transfected cells. Of note, treatment with VK_3 dramatically reduced cyclin D1, CDK4, Bcl-2 and Bcl-xL expression, in particular CDK4 and Bcl-xL expression, and induced strong activation of caspase-3 in Beclin 1-silenced cells. The data indicate that the knockdown of Beclin 1 did not significantly modulate cyclin D1, CDK4, Bcl-2 and Bcl-xL expression, but did significantly enhance VK_3-induced down-regulation of cyclin D1, CDK4, Bcl-2 and Bcl-xL expression and caspase-3 activation in SMMC-7721 cells *in vitro*.

Discussion

In this study, we examined the impact of Beclin 1 knockdown on spontaneous and VK_3-modulated hepatoma cell proliferation and autophagy in SMMC-7721 cells *in vitro*. A Beclin 1-specific siRNA approach was employed to successfully knockdown Beclin 1 expression in SMMC-7721 cells. The levels of Beclin 1 mRNA transcripts and proteins were reduced by 93 and 80%, respectively, in Beclin 1-specific siRNA-expressed cells, as compared to the vehicle-transfected cells. We found that the proliferation, viability and frequency of spontaneous apoptosis in Beclin 1-silenced cells were similar to those in vehicle-transfected control cells. Furthermore, the relative levels of cyclin D1, CDK4, Bcl-2 and Bcl-xL in Beclin 1-silenced cells were also comparable to those in control cells, accompanied by very low levels of active caspase-3 in both groups of cells. These data are consistent with previously reported results (19), and indicate that the knockdown of Beclin 1 expression did not significantly affect hepatoma cell proliferation and spontaneous apoptosis. However, our data were in disagreement with the finding that the partial down-regulation of Beclin 1 expression augmented the apoptosis of HepG2 cells *in vitro* (19). This difference may be due to the use of different hepatoma cell lines or different experimental conditions. The lack of significant regulatory effects of the knockdown of Beclin 1 expression on the proliferation and spontaneous apoptosis of human hepatoma cells suggests that Beclin 1 may be an upstream regulator of the development of hepatoma. Notably, the intensity of MDC staining in Beclin 1-silenced cells was significantly lower than that of the vehicle-transfected controls, indicating that the knockdown of Beclin 1 expression inhibited the spontaneous autophagy of hepatoma cells *in vitro*. Beclin 1 is a tumor suppressor and its deficient expression has been associated with the development of tumors in a clinical setting (22,23). The significant effect of Beclin 1 silencing on the inhibition of spontaneous hepatoma cell autophagy further demonstrates that Beclin 1 is a critical regulator of autophagy in human hepatoma cells (18).

VK_3 is a potent inducer of oxidative stress and has strong anti-tumor activities (24,25). VK_3 inhibition of the growth of tumor cells *in vitro* and *in vivo* (5-7) is associated with the induction of cell cycle arrest and caspase-dependent apoptosis (1,4). We examined the impact of Beclin 1 knockdown on VK_3-mediated growth inhibition, apoptosis and autophagy in human hepatoma cells, and found that treatment with VK_3 significantly inhibited the growth of SMMC-7721 cells and triggered a high frequency of hepatoma cell apoptosis *in vitro*, accompanied by high levels of caspase-3 activation. Indeed, VK_3 augments doxorubicin- and Fas-induced apoptosis in hepatoma-derived HepG2 cells (19). These regulatory effects of VK_3 were likely mediated by the inhibition of the expression of cyclin D1, CDK4, Bcl-2 and Bcl-xL in SMMC-7721 cells, similar to previous reports using other human hepatoma cell lines (4-7,19). Notably, treatment with VK_3 promoted hepatoma cell autophagy *in vitro*, similar to a previous report suggesting that VK2 promoted autophagy, thus inhibiting the growth of cholangiocellular carcinoma cells (8). Therefore, VK_3 may induce apoptosis and autophagy and mitigate the growth and survival of human hepatoma cells *in vitro*.

We found that Beclin 1-silenced SMMC-7721 cells were more sensitive to VK_3-induced apoptosis, but resistant to autophagy *in vitro*. Evidently, VK_3 treatment triggered a significantly higher frequency of Beclin 1-silenced SMMC-7721 cells undergoing apoptosis. Furthermore, VK_3 treatment dramatically reduced the expression of cyclin D1, CDK4, Bcl-2 and Bcl-xL expression in Beclin 1-silenced hepatoma cells, accompanied by higher levels of active caspase-3. Apparently, the knockdown of Beclin 1 expression enhanced the VK_3-induced hepatoma cell apoptosis *in vitro*. In addition, we found that the knockdown of Beclin 1 expression inhibited VK_3-induced autophagy in SMMC-7721 cells. These data support the notion that Beclin 1 is a positive regulator of autophagy, supporting survival and preventing apoptosis in many types of cells (14,22). Indeed, autophagy has been found to delay sulindac sulfide-induced apoptosis by sequestering mitochondrial death-promoting factors in colon cancer HT-29 cells (26). Furthermore, the inhibition of autophagy using siRNAs targeting genes that positively regulate autophagy or by treatment with inhibitors specific for autophagy, promotes apoptosis (11), and the inhibition of paraquat-induced autophagy accelerates the apoptotic process in neuroblastoma SH-SY5Y cells (27). It is possible that VK_3 or its associated signaling events may modulate the expression and activity of Beclin 1 and related signaling in hepatoma cells, promoting hepatoma cell autophagy. However, the precise mechanisms underlying the action of VK_3 during the process of autophagy remain to be further determined.

Beclin 1 was initially characterized as a Bcl-2-binding protein (28). Subsequent studies showed that Beclin 1 also interacts with Bcl-xL (19). Bcl-2/Bcl-xL inhibit apoptosis

by interfering with the action of pro-apoptotic proteins, and regulate autophagy by interacting with Beclin 1 (14). The interaction of Beclin 1 with either Bcl-2 or Bcl-xL may also be essential for the anti-apoptotic effects of Beclin 1 (18). We examined the effects of Beclin 1 silencing on VK_3-regulated Bcl-2 and Bcl-xL expression in SMMC-7721 cells. The levels of Bcl-2 and Bcl-xL expression were found to be significantly lower in Beclin 1-silenced SMMC-7721 cells than in the Beclin 1-expressing cells, indicating that Beclin 1 may protect the cells from VK_3-induced down-regulation of Bcl-2 and Bcl-xL expression in hepatoma cells. Given that VK_3 promoted strong autophagy in Beclin 1-expressing, but not in Beclin 1-silenced, SMMC-7721 cells, VK_3-promoted down-regulation of Bcl-2 and Bcl-xL expression may not be associated with its ability to promote autophagy in SMMC-7721 cells *in vitro*. Conceivably, Bcl-2 and Bcl-xL may have little effect on the regulation of VK_3-induced autophagy in SMMC-7721 cells. In addition, we observed that the knockdown of Beclin 1 expression enhanced VK_3-induced down-regulation of cyclin D1 and CDK4 expression, which are positive regulators of the cell cycle process. The down-regulation of cyclin D1 and CDK4 expression is usually related to cell cycle arrest, particularly at the G1 phase. Therefore, Beclin 1 may protect cyclin D1 and CDK4 expression from VK_3 down-regulation in SMMC-7721 cells *in vitro*, promoting the cell cycle and survival.

In conclusion, our study provides evidence that the knockdown of Beclin 1 expression induces resistance to spontaneous and VK_3-induced autophagy in SMMC-7731 cells *in vitro*. This supports the notion that Beclin 1 is a positive regulator of autophagy. Furthermore, the knockdown of Beclin 1 expression enhanced VK_3-induced apoptosis in SMMC-7721 cells, which was likely mediated by the down-regulation of the expression of cell cycle regulators cyclin D1 and CDK4 and anti-apoptotic Bcl-2 and Bcl-xL in SMMC-7721 cells. Conceivably, Beclin 1 may positively promote autophagy, preventing apoptosis in hepatoma cells. Therefore, our data suggest that the inhibition of autophagy may promote hepatoma cell apoptosis. Our findings may provide a basis for the design of novel therapeutic strategies for the treatment of hepatoma.

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